Am. J. Trop. Med. Hyg., 77(3), 2007, pp. 514–520

Development of a TaqMan®–Minor Groove Binding Protein Assay for the Detection and Quantification of Crimean-Congo Hemorrhagic Fever Virus

A. R. Garrison, S. Alakbarova, D. A. Kulesh, D. Shezmukhamedova, S. Khodjaev, T. P. Endy, and J. Paragas*

Virology Division and Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland; Institute of Virology, Ministry of Health, Tashkent, Uzbekistan

Abstract. Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus of the genus *Nairovirus* and the family *Bunyaviridae*. It is a negative-strand RNA virus comprised of small (S), medium (M), and large (L) genome segments. The S segment encodes for nucleocapsid protein, the M segment codes for envelope glycoproteins (Gn and Gc), and the L segment codes for the RNA-dependent RNA polymerase. Currently, there are a limited number of methods for rapidly diagnosing CCHFV infections. We developed a real-time, reverse transcription–polymerase chain reaction assay for the rapid detection of CCHFV by using the TaqMan®–minor groove binding protein probe technology. The primers and probes were designed to amplify and detect a region in the S segment of CCHFV that is conserved across multiple strains. The limit of detection of the assay was 10 genome copies of RNA. This primer and probe set was specific to 18 strains of CCHFV tested and did not cross-react with either a DNA panel of 78 organisms or a panel of 28 diverse RNA viruses. This will rapidly and specifically detect CCHFV, and it has been used to detect CCHFV infection in samples from humans, animals, and ticks.

INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus of the genus *Nairovirus* in the family *Bunyaviridae*.[1] This virus is endemic in sub-Saharan Africa, the Middle East, and southern Eurasia. Transmission to humans is primarily through the bite of an infected Ixodid tick, most commonly of the *Hyalomma* genus, or direct contact with blood or tissues from infected humans or livestock.[2–4] In humans, infection with CCHFV can develop into a hemorrhagic fever with substantial morbidity and a reported mortality rate from 10% to 50%[5,6] There is a significant risk for nosocomial infection that may result in a more severe illness with a higher mortality rate.[7–9]

This virus is negative-sense, single-stranded RNA virus with a genome of 17,100–22,800 nucleotides. The genome contains three segments: small (S), medium (M), and large (L). The tripartite genome encodes a nucleocapsid protein (S segment 1,700–2,100 nucleotides), envelope glycoproteins Gn and Gc (M segment 4,400–6,300 nucleotides), and a viral RNA-dependent polymerase (L segment 11,000–14,000 nucleotides).[10–12] There is extensive genetic diversity across the strains of CCHFV within the S and M segments, with little data available on the L segment. Thirteen full-length CCHF genomes were completely sequenced and reported in Genbank.[13–16] This diversity illustrates the need for a broad-range detection assay specific to the CCHFV species.

Human diagnosis of CCHF to date has relied on several methods. Clinical observations and patient history are useful, but cannot be used to distinguish other diseases with hemorrhagic symptoms. Virus isolation using cell culture or intracranial or intraperitoneal injection of newborn mice methods require the use of a biosafety level 4 laboratory, and without downstream diagnostics, isolation is a limited tool. Antibody tests, both enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody assay, are limited because IgG and IgM are not detectable until approximately seven days after the onset of illness, and antibodies are rarely detectable in fatal cases of CCHF.[5,15,17] There is also the problem of cross-reactivity with other members of the *Nairovirus* family using ELISA.[18] Several reverse transcription–polymerase chain reaction (RT-PCR) protocols have been reported, but analysis is limited by the need to amplify the product to levels that may be visualized after electrophoresis on an agarose gel, which is limited by the input RNA.[17,19] These protocols require a two-step amplification process followed by gel electrophoresis, which increase the labor intensity and time to outcome. Two one-step real-time RT-PCR assays have also been reported for diagnosing CCHF. One assay is specific to strains circulating in the Balkan region,[20] and the second assay was developed using strains with a wide geographic distribution.[21] The high mortality rate and high risk for nosocomial infection along with widespread geographic distribution of CCHFV with sporadic outbreaks prompt the need for a rapid and specific presumptive diagnostic assay against numerous strains.

The objective of this study was to develop a one-step, real-time RT-PCR for the rapid and specific detection the majority of known CCHFV strains. The technologies of a Taq-Man®–minor groove binder (MGB) probe and real-time RT-PCR were incorporated for the combined speed, sensitivity, and specificity. The Taq-Man®-MGB probe has an MGB ligand and a nonfluorescent quencher conjugated to the 3′-end and a fluorescent reporter dye at the 5′-end. The MGB ligand allows for the use of shorter and more specific probes by increasing the stability of the hybridization of the probe to the target.[22,23] The assay was used to confirm several suspected CCHF cases in Uzbekistan.

MATERIALS AND METHODS

RT-PCR primers, TaqMan®-MGB probe, and target sequences. The primers were designed to a consensus sequence

* Address correspondence to J. Paragas, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 33 North Drive, Room 2E19A, Bethesda, MD 20892. E-mail: paragasj@niaid.nih.gov

Report Documentation Page			*Form Approved* *OMB No. 0704-0188*
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.			
1. REPORT DATE **1 SEP 2007**	2. REPORT TYPE **N/A**		3. DATES COVERED **-**
4. TITLE AND SUBTITLE **Development of a Taqman(R) minor groove binding protein assay for the detection and quantification of Crimean-Congo hemorrhagic fever. American Journal of Tropical Medicine and Hygiene 77:514-520.**			5a. CONTRACT NUMBER
			5b. GRANT NUMBER
			5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) **Garrison AR, Alakbarova S, Kulesh DA, Shezmukhamedova D, Khodjaev S, Endy TP, Paragas J.**			5d. PROJECT NUMBER
			5e. TASK NUMBER
			5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD**			8. PERFORMING ORGANIZATION REPORT NUMBER **TR-06-133**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)			10. SPONSOR/MONITOR'S ACRONYM(S)
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)
12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release, distribution unlimited**			
13. SUPPLEMENTARY NOTES			

14. ABSTRACT

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus of the genus Nairovirus and the family Bunyaviridae. It is a negative-strand RNA virus comprised of small (S), medium (M), and large (L) genome segments. The S segment encodes for nucleocapsid protein, the M segment codes for envelope glycoproteins (Gn and Gc), and the L segment codes for the RNA-dependent RNA polymerase. Currently, there are a limited number of methods for rapidly diagnosing CCHFV infections. We developed a real-time, reverse transcription-polymerase chain reaction assay for the rapid detection of CCHFV by using the TaqMan((R))-minor groove binding protein probe technology. The primers and probes were designed to amplify and detect a region in the S segment of CCHFV that is conserved across multiple strains. The limit of detection of the assay was 10 genome copies of RNA. This primer and probe set was specific to 18 strains of CCHFV tested and did not cross-react with either a DNA panel of 78 organisms or a panel of 28 diverse RNA viruses. This will rapidly and specifically detect CCHFV, and it has been used to detect CCHFV infection in samples from humans, animals, and ticks.

15. SUBJECT TERMS

Crimean-Congo hemorrhagic fever, CCHF, diagnosis, identification, Taqman, methods, cross-reaction potential

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**SAR**	**7**	

of the S segment of all documented CCHFV isolates (GenBank), with the maximum conservation across all of the sequences. The basepair ranges for the primers and probe are in reference to the S segment of the IbAr 10200 strain (GenBank accession U88410), and the primer and probe sequences contain several basepair substitutions based on the consensus. As shown in Figure 1A, the forward primer, designated CCHF Forward, is 5′-GGA GTG GTG CAG GGA ATT TG-3′ and is complementary to the positive RNA sense strand from nucleotide positions 649 to 668. The reverse primer, designated CCHF Reverse, is 5′-CAG GGC GGG TTG AAA GC-3′ and is complementary to the negative RNA sense strand from nucleotide positions 689 to 705. The TaqMan®-MGB probe, designated CCHF-N Probe, is 5′-6FAM-CAA GGC AAG TAC ATC A T-MGBNFQ-3′, is complementary to the sense RNA strand from nucleotide positions 670 to 687. The resulting single RT-PCR amplicon is 57 basepairs.

Viral RNA propagation and RNA isolation. The 18 CCHFV isolates used for the evaluation of the CCHF real-time RT-PCR assay were obtained from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) collection (Table 1). SW-13 (human adrenal gland carcinoma; ATCC CLL-105) in T75 flasks were propagated in Eagle's minimum essential medium with Earle's salts (EMEM) containing 10% fetal bovine serum (FBS), 4% non-essential amino acids, 1% L-glutamine, 1% penicillin, and 1% streptomycin. For infection, the EMEM was removed and 1 mL of 4×10^5 plaque-forming units (PFU) of virus in EMEM, with the same contents as previously described, was added. After infection for one hour at 37°C in an atmosphere of with 5% CO_2,15 mL of the same media with only 2% FBS was added. After infection, the flasks were incubated at 37°C for 3–4 days, until a 4+ cytopathic effect was observed. The supernatant was then harvested, cellular debris was removed by centrifugation, and the supernatant was stored at −70°C. Total RNA was extracted from 18 CCHFV isolates using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For isolating viral RNA to determine the limit of infection, Trizol LS (Invitrogen, Carlsbad, CA) reagent was used according to the manufacturer's instructions. All CCHFV work was performed in a class II hood in a biosafety level 4 (BSL-4) laboratory at USAMRIID (Fort Detrick, MD). All personnel wore positive-pressure protected suits (ILC Dover, Fredrica, DE) supplied with umbilical-fed air.

Real-time RT-PCR assay. For the one-step real-time RT-PCR for CCHFV, 5 μL of total RNA was added to 15 μL of a master mixture by using the Superscript™ One-Step RT-PCR with Platinum Taq kit (Invitrogen). The final master mixture was contained Invitrogen 1× Reaction Mixture (with 0.02 mM of each dNTP), 4.95 mM $MgSO_4$, 250 ng of bovine serum albumin, 1.25 μM each of CCHF Forward and CCHF Reverse primers, 100 nM CCHF-N probe, and 0.25 units per reaction of SSII RT/Platinum® Taq Mixture. The 20-μL reactions were added to LightCycler capillary tubes (Roche Diagnostics, Indianapolis, IN) and run on the Ruggedized Advanced Pathogen Identification Device (RAPID) (Idaho Technology Inc., Salt Lake City, UT.). The cycling conditions used were 50°C for 15 minutes to synthesize cDNA, followed by 1 cycle at 95°C for 5 minutes to inactivate the SSII RT and activate the Platinum® *Taq* polymerase, and 45 cycles at 94°C for 1 second and 55°C for 20 seconds, and 68°C for 5 seconds for amplification. Fluorescence was read in Channel 1 at the end of each 68°C step. Analysis of the real-time RT-PCR data was performed with the LightCycler software version 3.5.3 (Roche Diagnostics).

DNA and RNA panel evaluations. The assay was tested against two panels for cross-reactivity. The RNA cross-reactivity panel is shown in Table 2. The DNA and RNA samples in each panel were tested at a concentration of 100 pg/μL, as determined by determining the optical density at 260 nm (OD_{260}). The CCHFV strains that were tested are listed in Table 1.

Limit of detection. A dilution series of CCHFV UG 3010 extracted from cell culture supernatant was used to determine

A

10200 Reference	Forward Primer		TaqMan-MGB Probe		Reverse Primer
649-705bp	GGAGTGGTGCAGGGAATTTG	T	CAAAGGCAAGTACATCAT	G	GCTTTCAACCCGCCCTG

B

HY13	GGAGTGGTGCAGGGAATTTGTCAAAGGCAAGTACATCATGGCTTTCAACCCGCCCTG
IbAr 10200	AGACTGGTGCAGGGAGTTTGTCAAAGGCAAATACATCATGGCCTTCAACCCACCATG
DAK 8194	AGAATGGTGCAGGGAGTTTGTCAAGGGCAAATATATAATGGCCTTCAATCCACCCTG
SPU 41/84*	GGACTGGTGCAGGGAGTTTGTCAAGGGCAAATACATCATGGCATTCAACCCACCATG
SPU 415/85	AGACTGGTGCAGGGAGTTTGTCAAAGGCAAATACATCATGGCCTTCAACCCACCATG
SPU 115/87*	GGACTGGTGCAGGGAGTTTGTCAAAGGCAAATACATCATGGCCTTCAACCCACCGTG
SPU 128/81/7	GGACTGGTGCAGGGAGTTTGTCAAAGGCAAATACATCATGGCCTTCAACCCACCATG
SPU 134/87*	GGACTGGTGCAGGGAGTTCGTCAAGGGCAAATACATCATGGCCTTCAACCCACCATG
SPU 97/85*	AGACTGGTGCAGGGAGTTTGTCAAGGGCAAATACATCATGGCCTTCAACCCACCATG
UG 3010	GGAATGGTGCAGGGAATTTGTCAAGGGCAAGTACATCATGGCTTTCAACCCACCCTG

FIGURE 1. **A**, Primers and probe used in the Crimean-Congo hemorrhagic fever virus (CCHFV) assay, which are shown as the leading strand for alignment purposes. bp = basepairs; MGB = minor groove binding protein. **B**, Alignment of 10 of the CCHF isolates used in this study. * indicates sequences that were not available on GenBank. Therefore, an area of the small segment was sequenced for several of the strains to determine the similarity to the primers and probe used in the real-time reverse transcription–polymerase chain reaction assay.

TABLE 1

Eighteen CCHFV isolates tested to determine the detectable range of isolates that can be amplified with the real-time CCHF RT-PCR assay*

Isolate	Source	Source location	Year collected	Real-time RT-PCR assay results	% Similarity to primers and probe
Hy13	*Hyalomma asiaticum* tick	China	1968	+	100
M-20	Human	Nigeria	–	+	ND
IbAr 10200		Nigeria	–	+	87
DAK 8194	Human	Senegal	–	+	82
SPU 41/84	Human	South Africa	–	+	88
SPU 415/85	Human	South Africa	–	+	93
SPU 115/87	Human	South Africa	–	+	89
SPU 128/81/7	Tick	South Africa	–	+	89
SPU 134/87	Human	South Africa	–	+	86
SPU 97/85	Human	South Africa	–	+	86
SPU 94/85	Human	South Africa	–	+	ND
UG 3010	Human	Uganda	–	+	96
I 40	Human	Uzbekistan	1983	+	ND
I 5415	Tick	Uzbekistan	1978	+	ND
2219 KKK28	*Alveonasus lahorensis* tick	Uzbekistan	1981	+	ND
I-35	Human	Uzbekistan	1982	+	ND
I-248	Human	Uzbekistan	1989	+	ND
I-313	Human	Uzbekistan	2002	+	ND

* Shown are corresponding viral source information (if documented), as well as the sequence similarity to the primers and probe. All isolates were detectable using 100 pg of RNA in the real-time assay. CCHFV = Crimean Congo hemorrhagic fever virus; RT-PCR = reverse transcription–polymerase chain reaction; ND = not determined.

the limit of detection (LOD) of total RNA, and the ability of the assay to be quantitative (Figure 2). The concentration of total RNA was determined in duplicate determining the OD_{260}. The stock RNA was then used to make 10-fold serial dilutions from 100 pg to 1 ag in DNase/RNase–free water. The dilution series was run in duplicate on the real-time assay. The LOD of PFU of virus was established using IbAr 10200 RNA extracted from cell culture supernatant. A 10-fold dilution series from 2,333 PFU to 2 PFU of IbAr 10200 RNA was used with the assay in triplicate and repeated.

TABLE 2

Viral RNA species tested for cross-reactivity with the CCHF real-time RT-PCR assay*

Sample	Reactivity
Venezuelan equine encephalitis (VEE) IA/B (Trinidad donkey)	–
VEE IC C0951006	–
VEE ID V209A	–
VEEIE 68U201	–
VEE IF 78V3531	–
VEE II Everglades	–
VEE IIIA Mucambo	–
VEE IV	–
VEE V	–
VEE VI	–
Western equine encephalitis (WEE) B-11	–
WEE CBA	–
WEE OR17	–
Eastern equine encephalitis (EEE) Georgia '99	–
EEE Arg LL	–
EEE 76V-25343	–
Highlands J	–
Semliki Forest virus	–
Getah Amm 2021	–
Mayaro BeH256	–
Middleburg	–
Ndumu	–
Barman Forest	–
Sinbis UgMP 6440	–
CCHF U 3010 positive control	+

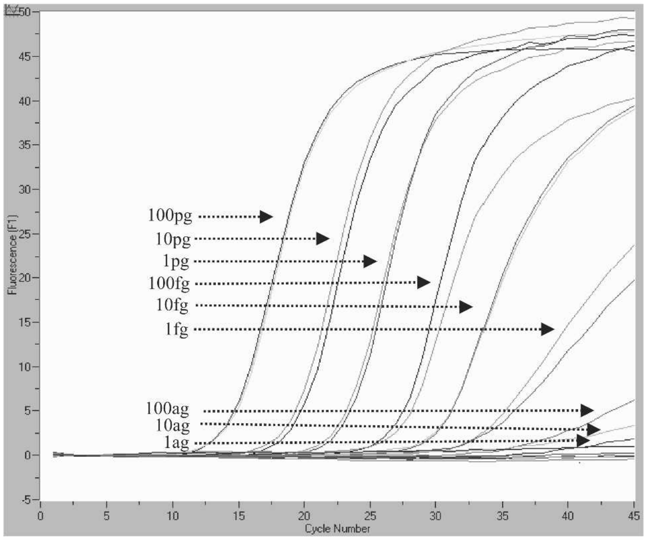
* Each RNA sample was tested at a total RNA concentration of 100 pg. CCHF U3010 was included as a positive control with all tests. All samples were negative in the assay. For definitions of abbreviations, see Table 1.

A standard curve was produced with an 80-basepair synthesized antisense RNA oligonucleotide (Dharmaco Inc., Lafayette, CO). The standard RNA oligonucleotide sequence was 5′-GAU GUC CCC CCA GGG CGG GUU GAA AGC CAU GAU GUA CUU GCC UUU GAC AAA UUC CCU GCA CCA CUC CAC AUG UUC ACG GC-3′. To determine the LOD of the CCHFV real-time RT-PCR assay, a 10-fold dilution series ranging from 1.18×10^6 to 0.118 genome copies of the standard RNA oligonucleotide was used in triplicate and repeated.

Indirect hemagglutination (HI), agarose gel diffusion and precipitation (AGDP), and complement fixation (CF). These procedures were performed at the Institute of Virology, Ministry of Health, (Tashkent, Uzbekistan) as previously described.[24]

Sequencing the CCHF S segment. The sequence data for the SPU 41/84, SPU 115/87, SPU 134/87, and SPU 97/85 South African strains of CCHF listed in Table 1 are not available. A 354-basepair region of the CCHF S segment was sequenced to determine its similarity to the real-time RT-PCR primers and probe. The forward and reverse primer sequences used were 5′-GGT TGT CCG TGT CAA TGC-3′ and 5′-CCT CTA CAG TCT TTT TGG C-3′, respectively. The cDNA for sequencing was amplified from total viral RNA using the Superscript™ One-Step RT-PCR with Platinum Taq kit (Invitrogen). The master mixture contained 10 μL (10 pg–1 μg) of viral RNA and Invitrogen 1× Reaction Mixture (with 0.02 mM of each dNTP), 4.95 mM $MgSO_4$, 0.2 μM each of the CCHF Forward and CCHF Reverse primers, and 0.25 units per reaction of SSII RT/Platinum® Taq Mix. The cycling conditions used were 50°C for 30 minutes to synthesize cDNA, followed by 1 cycle at 95°C for 2 minutes to inactivate the SSII RT and activate the Platinum® *Taq* polymerase, and 45 cycles at 94°C for 15 seconds and 51°C for 30 seconds, and 72°C for 1 minute for amplification. The amplified products were purified by electrophoresis on a 1% agarose gel followed by extraction using with QIAquick Gel extraction (Qiagen). The sequencing was performed using Big Dye Terminator version 3.1 (Applied Biosystems, Foster City, CA) following the manufacturer's instructions.

A



B

Total RNA Concentration	CT value (Average)	Replicates Detected
100pg	14.3	2
10pg	19.2	2
1pg	22.9	2
100fg	27.2	2
10fg	30.3	2
1fg	33.4	2
100ag	33.6	1
10ag	35.8	1
1ag	37.2	1

FIGURE 2. Amplification of a 10-fold dilution series of total RNA extracted from Crimean-Congo hemorrhagic fever virus strain UG 3010 using the real-time reverse transcription–polymerase chain reaction as described in the Materials and Methods. **A**, Ruggedized advanced pathogen identification device screen display of fluorescence output versus cycle number. **B**, The RNA concentrations, cycle threshold (Ct) values, and number of replicates detected shown in **A**.

RESULTS

Real-time RT-PCR design. The S segment of CCHF has the most complete sequencing data available for strains characterized to date. Sequences of the CCHF S segments were aligned using the Clustal W method. The area containing the most conservation was chosen, with a desired amplicon size of less than 250 basepairs. Because of the limited conservation of the consensus, a small region was selected that contained the desired parameters for optimal primer design and reasonable sequence conservation (Figure 1B).

The primer and probe sequences are shown in Figure 1A in the leading strand orientation. The primers and Taq-Man-MGB probe were designed in close proximity with one base between each primer and the probe. The final amplicon was 57 basepairs, as shown in Figure 1A.

Cross-reactivity and specificity testing. All 18 CCHFV isolates shown in Table 1 were detected by using the CCHFV real-time RT-PCR assay. The isolates listed included those from numerous virus genetic groupings as described by Deyde and others.[13] Sequence similarity to the primers and probe ranged from 82% to 100%, and all were amplifiable by the real-time RT-PCR assay. Most of the viral isolates tested were logarithmically amplified in the assay, which may be influenced by the quality of the RNA extracted.

The assay was blindly tested for cross reactivity against a bacterial DNA panel, as described by Kulesh and others,[25] and a viral RNA panel derived from purified USAMRIID bacterial and viral stocks. The RNA panel included viruses from within the genus *Nairovirus* and some in the family *Bunyaviridae* to ensure the specificity of the primer and probe set to CCHF. All DNA and RNA samples were tested using 100 pg of material. The results in Table 2 show that the real-time CCHF RT-PCR assay does not cross-react with any of the viral species tested. The assay did not cross-react with any of the bacterial DNA tested. The lack of false-positive results in the other members of the genus *Nairovirus* indicates the specificity of the assay for CCHF.

Determination of LOD. The primer and TaqMan®-MGB probe were tested for LOD with total viral RNA against CCHFV strains UG 3010 and IbAr 10200, which have sequence homologies of 96% and 87% to the CCHF specific primers and probes, respectively (Table 1). The assay was reproducibly able to logarithmically amplify 1 fg of total viral RNA for UG3010 (Figure 2). The LOD for extracted IbAr 10200 RNA was 23.4 PFU. To establish a LOD for gene copies of the S segment RNA, a dilution series of the standard RNA oligonucleotide was extensively tested (Figure 3). We obtained an LOD of 11.8 genome copies amplifying a minimum of two of three replicates.

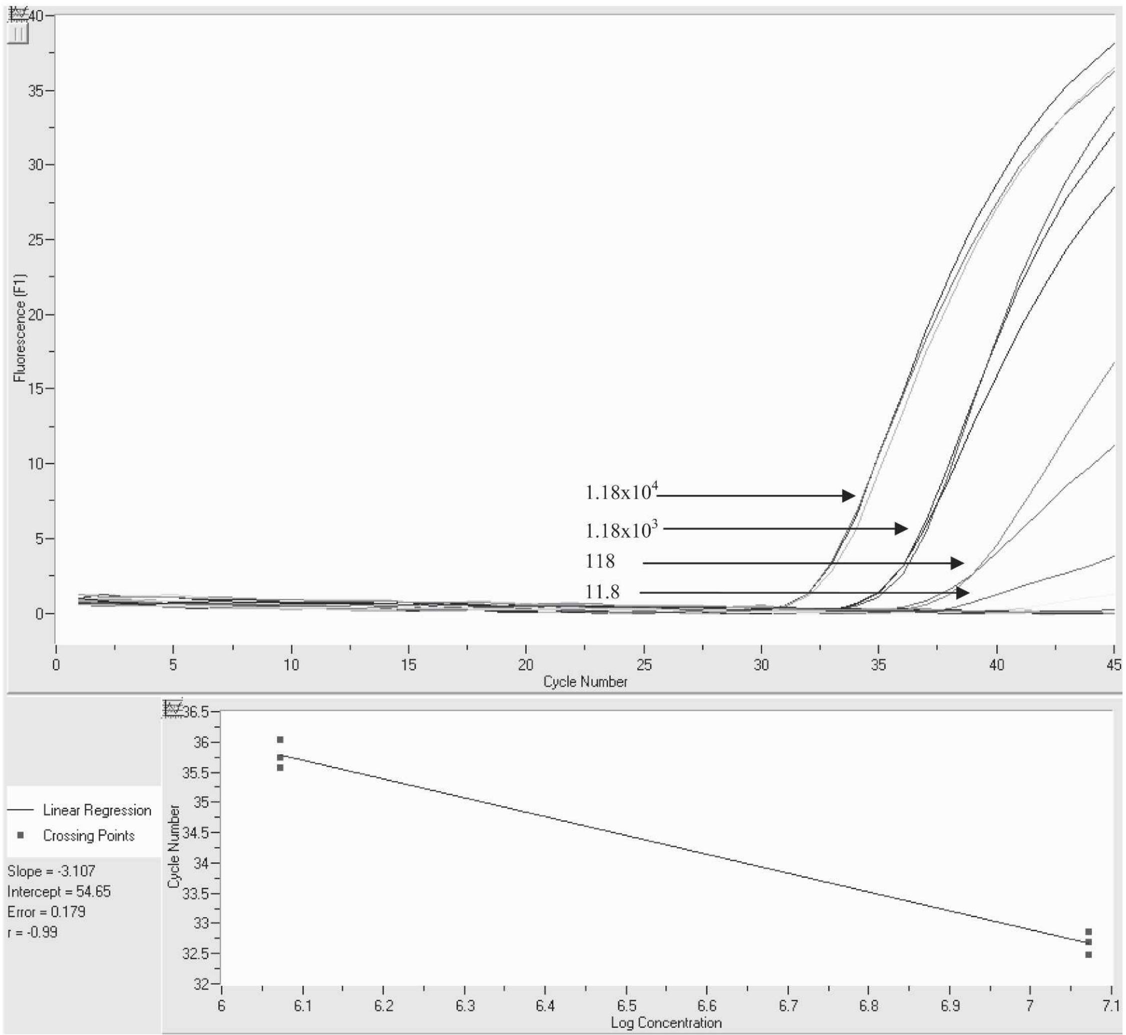
The primary assessment for CCHFV in a sample was made by the software included with the RAPID. A secondary analysis was performed by the user reviewing the cycle threshold (Ct) values and amplification curves for each sample. Those samples that do not amplify prior to 40 cycles are considered negative. Those samples that have a Ct value close to 40 with a non-linear curve are considered undetermined and should be retested.

Application of the assay to human samples. As shown in Table 3, the real-time assay confirmed several suspected fatal human cases of CCHF. The initial diagnosis was made from clinical observation and by laboratory tests. The laboratory tests included one or more of the following: HI, AGDP, and CF. Using archived and unidentified samples, viral RNA was extracted from the patient's plasma and that of multiple familial and hospital contacts. All samples were coded for blinded and unlinked analysis. The deceased patient samples were identified as positive by the real-time RT-PCR assay, which supports the cause of death. The contacts were monitored for secondary infection and remained negative by all laboratory tests.

DISCUSSION

The real-time RT-PCR assay described in this report was developed to detect the S segment of CCHFV, despite the great genetic variability within this virus. The S segment was chosen because it has the most complete nucleotide sequence database in GenBank. The primers and probes were designed

A



B

RNA Concentration	Calculated RNA Concentration	CT value (Average)	Replicates Detected
$1.18x10^4$	$1.18x10^4$	32.4	3
$1.18x10^3$	$1.19x10^3$	35.5	3
118		37.8	2
11.8		39.1	2

FIGURE 3. **A**, Limit of detection analysis of synthesized standard 80-basepair RNA oligonucleotide. The lower panel shows the regression analysis based on the four dilutions in the upper panel, showing the linearity of the assay. Points < 11.8 gene copies lose linearity. **B**, The RNA concentrations, cycle threshold (Ct) values, and number of replicates detected shown in **A**.

to the most homologous target from an alignment of 81 strains of the S segment of CCHFV. The conserved region was limited in size; thus, use of a TaqMan®-MGB probe produced a short probe sequence with high specificity and stable hybridization to the target. In addition, exact basepair complements at the 3′ end of the primers and probe were critical to ensure amplification of strains that contained multiple basepair changes within those regions.

The original amplification strategy of the assay contained two steps: a 94°C denaturation step and a 60°C annealing and elongation step. The assay has the ability to detect a wide range of strains of CCHFV including strains from virus genetic groups I–IV as described by Deyde and others.[13] Several of the strains tested contained multiple basepair changes in the probe and reverse primer area; some of these changes significantly decreased the melting temperature within the target area. These strains displayed a linear amplification on the fluorescence versus cycle number readout. By decreasing the annealing temperature to 55°C, and adding an elongation step at 68°C, these strains amplified logarithmically. The

TABLE 3
Information on patients with CCHF from Uzbekistan*

Patient information

Sample name	Year of disease	Days from exposure to hospitalization	Hospital duration day	Blood sample collection day post-infection	Death day post-infection	Suspected source of CCHF infection
U-248	1998	2	5	+7	+7	Contact with CCHF infected patient's blood
U-368	2004	5	2	+6	+7	Tick
U-356	2004	7	1	+8	+8	Tick

Laboratory test results

Sample name	Sample type	HI results	AGDP	CF	Real-time RT-PCR results
U-248	SMB isolate of human sample	Positive	Positive	Positive	Positive
U-368	Human sera	Negative	Negative	Negative	Positive
U-356	Human sera	Positive	ND	ND	Positive

* SMB = suckling mouse brain; HI = hemagglutination inhibition; AGDP = agarose gel diffusion and precipitation; CF = complement fixation; ND = not done. For definitions of other abbreviations, see Table 1.

CCHFV real-time RT-PCR assay detected a broad range of CCHFV strains from various geographic regions, without losing specificity to the species. However, because of the nucleotide variability among strains of CCHFV,[13,26] sequencing or multiple assays to regional variants would be needed for further strain determination. New probe technologies are being developed using the variance in melting temperature to detect single basepair changes among the viral strains.[27] With further development and validation of these probe technologies and a more complete database of sequences for the S, M, and L segments of CCHFV, the ability for broad detection and specific strain determination within a single assay could be possible.

To create a quantitative assay, the use of a standard with a known gene copy concentration was necessary. Using transcribed RNA from a plasmid containing the S segment of CCHFV was rejected because of the inability to completely eliminate low levels of plasmid DNA, which was determined by performing the assay without reverse transcriptase in which only DNA would be amplified. The use of total RNA extracted from a CCHFV stock derived from a cell culture supernatant cannot be used as a standard because of possible contamination with cellular RNA. In addition, the total RNA could not be used to determine the viral genomes contained in a sample because the molarity of the total RNA is unknown. Because of the short target, a commercially synthesized RNA oligonucleotide could be used as a quantifying standard. A regressional analysis performed on the real-time RT-PCR of the synthesized RNA showed that 1.18×10^6 to 11.8 gene copies were linear, with an r value of −1. Adding values below 11.8 gene copies reduced the r value to −0.96; therefore, with fewer than 11.8 gene copies, the assay lost linearity and the detection of viral RNA below this threshold was unreliable. With the use of a standard curve, the CCHFV real-time RT-PCR assay was used to accurately measure the genome copies of samples containing the virus. This quantification would be particularly useful in the study of CCHF disease course in animals, for animal model development, and therapeutic studies. Viral load would also be useful for determining viral clearance in an infected individual.

The assay described herein has been used to evaluate real world samples. Samples from probable CCHFV human infections were tested and supported results of other laboratory tests. Clinical diagnosis also supported a cause of CCHFV infection. This suggests that the assay may have important applications in the diagnosis of CCHFV infections, along with being an important tool for veterinary testing.

In conclusion, a presumptive diagnostic determination using the CCHFV real-time RT-PCR assay, from RNA isolation to RT-PCR outcome, can be reported in less than three hours. This would be valuable as a tool to evaluate the need for immediate isolation of a patient with a potential viral hemorrhagic fever infection. A patient's illness would need to be confirmed in conjunction with classic methods of diagnostics, such as virus isolation and immunohistochemistry, but early detection is critical in preventing nosocomial infections and assessing possible exposure to the community. This assay can also be used for a long-term monitoring program to assess the viremia and the number of infected ticks and animal reservoirs in disease-endemic regions. This type of surveillance will aid in the establishment of background levels of viremia maintained in a vector and/or reservoir population. This surveillance program would be critical for advanced notice of an increase in viral activity in a region. Notification of the public to increase protective measures from tick and animal contact could potentially decrease the number of human outbreaks in disease-endemic regions.

Received September 22, 2006. Accepted for publication December 22, 2006.

Financial support: This study was supported in part by the U.S. Defense Threat Reduction Agency (DTRA) and administered by the U.S. Civilian Research and Development Foundation for the Independent States of the Former Soviet Union (CRDF).

Disclaimer: Any opinions, interpretations, conclusions, and recommendations expressed in this material are those of the author(s) and do not necessarily reflect those of the DTRA, the CRDF, or the U.S. Army.

Authors' addresses: A. R. Garrison and T. P. Endy, Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, 21702-5011. S. Alakbarova and D. Shezmukhamedova, Institute of Virology, Ministry of Health, Tashkent, Uzbekistan. D. A. Kulesh, Diagnostic Systems Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, 21702-5011. J. Paragas, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 33 North Drive, Room 2E19A, Bethesda, MD 20892, Telephone: 301-443-5626, Fax: 301-480-3213, E-mail: paragasj@niaid.nih.gov.

REFERENCES

1. Swanepoel R, Leman PA, Burt FJ, Jardine J, Verwoerd DJ, Capua I, Bruckner GK, Burger WP, 1998. Experimental infection of ostriches with Crimean-Congo haemorrhagic fever virus. *Epidemiol Infect 121:* 427–432.
2. Dohm DJ, Logan TM, Linthicum KJ, Rossi CA, Turell MJ, 1996. Transmission of Crimean-Congo hemorrhagic fever virus by *Hyalomma impeltatum* (Acari:Ixodidae) after experimental infection. *J Med Entomol 33:* 848–851.
3. Rodriguez LL, Maupin GO, Ksiazek TG, Rollin PE, Khan AS, Schwarz TF, Lofts RS, Smith JF, Noor AM, Peters CJ, Nichol ST, 1997. Molecular investigation of a multisource outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates. *Am J Trop Med Hyg 57:* 512–518.
4. Swanepoel R, Shepherd AJ, Leman PA, Shepherd SP, McGillivray GM, Erasmus MJ, Searle LA, Gill DE, 1987. Epidemiologic and clinical features of Crimean-Congo hemorrhagic fever in southern Africa. *Am J Trop Med Hyg 36:* 120–132.
5. Burt FJ, Leman PA, Abbott JC, Swanepoel R, 1994. Serodiagnosis of Crimean-Congo haemorrhagic fever. *Epidemiol Infect 113:* 551–562.
6. Hoogstraal H, 1979. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. *J Med Entomol 15:* 307–417.
7. Burney MI, Ghafoor A, Saleen M, Webb PA, Casals J, 1980. Nosocomial outbreak of viral hemorrhagic fever caused by Crimean Hemorrhagic fever-Congo virus in Pakistan, January 1976. *Am J Trop Med Hyg 29:* 941–947.
8. Papa A, Bino S, Llagami A, Brahimaj B, Papadimitriou E, Pavlidou V, Velo E, Cahani G, Hajdini M, Pilaca A, Harxhi A, Antoniadis A, 2002. Crimean-Congo hemorrhagic fever in Albania, 2001. *Eur J Clin Microbiol Infect Dis 21:* 603–606.
9. Shepherd AJ, Swanepoel R, Shepherd SP, Leman PA, Blackburn NK, Hallett AF, 1985. A nosocomial outbreak of Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part V. Virological and serological observations. *S Afr Med J 68:* 733–736.
10. Aitichou M, Saleh SS, McElroy AK, Schmaljohn C, Ibrahim MS, 2005. Identification of Dobrava, Hantaan, Seoul, and Puumala viruses by one-step real-time RT-PCR. *J Virol Methods 124:* 21–26.
11. Honig JE, Osborne JC, Nichol ST, 2004. Crimean-Congo hemorrhagic fever virus genome L RNA segment and encoded protein. *Virology 321:* 29–35.
12. Kinsella E, Martin SG, Grolla A, Czub M, Feldmann H, Flick R, 2004. Sequence determination of the Crimean-Congo hemorrhagic fever virus L segment. *Virology 321:* 23–28.
13. Deyde VM, Khristova ML, Rollin PE, Ksiazek TG, Nichol ST, 2006. Crimean-Congo hemorrhagic fever virus genomics and global diversity. *J Virol 80:* 8834–8842.
14. Hewson R, Gmyl A, Gmyl L, Smirnova SE, Karganova G, Jamil B, Hasan R, Chamberlain J, Clegg C, 2004. Evidence of segment reassortment in Crimean-Congo haemorrhagic fever virus. *J Gen Virol 85:* 3059–3070.
15. Whitehouse CA, 2004. Crimean-Congo hemorrhagic fever. *Antiviral Res 64:* 145–160.
16. Yashina L, Petrova I, Seregin S, Vyshemirskii O, Lvov D, Aristova V, Kuhn J, Morzunov S, Gutorov V, Kuzina I, Tyunnikov G, Netesov S, Petrov V, 2003. Genetic variability of Crimean-Congo haemorrhagic fever virus in Russia and Central Asia. *J Gen Virol 84:* 1199–1206.
17. Drosten C, Kummerer BM, Schmitz H, Gunther S, 2003. Molecular diagnostics of viral hemorrhagic fevers. *Antiviral Res 57:* 61–87.
18. Burt FJ, Spencer DC, Leman PA, Patterson B, Swanepoel R, 1996. Investigation of tick-borne viruses as pathogens of humans in South Africa and evidence of Dugbe virus infection in a patient with prolonged thrombocytopenia. *Epidemiol Infect 116:* 353–361.
19. Burt FJ, Leman PA, Smith JF, Swanepoel R, 1998. The use of a reverse transcription-polymerase chain reaction for the detection of viral nucleic acid in the diagnosis of Crimean-Congo haemorrhagic fever. *J Virol Methods 70:* 129–137.
20. Duh D, Saksida A, Petrovec M, Dedushaj I, Avsic-Zupanc T, 2006. Novel one-step real-time RT-PCR assay for rapid and specific diagnosis of Crimean-Congo hemorrhagic fever encountered in the Balkans. *J Virol Methods 133:* 175–179.
21. Yapar M, Aydogan H, Pahsa A, Besirbellioglu BA, Bodur H, Basustaoglu AC, Guney C, Avci IY, Sener K, Setteh MH, Kubar A, 2005. Rapid and quantitative detection of Crimean-Congo hemorrhagic fever virus by one-step real-time reverse transcriptase-PCR. *Jpn J Infect Dis 58:* 358–362.
22. Afonina I, Zivarts M, Kutyavin I, Lukhtanov E, Gamper H, Meyer RB, 1997. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res 25:* 2657–2660.
23. Afonina IA, Reed MW, Lusby E, Shishkina IG, Belousov YS, 2002. Minor groove binder-conjugated DNA probes for quantitative DNA detection by hybridization-triggered fluorescence. *Biotechniques 32:* 940-944, 946–949.
24. Saidi S, Casals J, Faghih MA, 1975. Crimean hemorrhagic fever-Congo (CHF-C) virus antibodies in man, and in domestic and small mammals, in Iran. *Am J Trop Med Hyg 24:* 353–357.
25. Kulesh DA, Baker RO, Loveless BM, Norwood D, Zwiers SH, Mucker E, Hartmann C, Herrera R, Miller D, Christensen D, Wasieloski LP Jr, Huggins J, Jahrling PB, 2004. Smallpox and pan-orthopox virus detection by real-time 3′-minor groove binder TaqMan assays on the roche LightCycler and the Cepheid smart Cycler platforms. *J Clin Microbiol 42:* 601–609.
26. Yashina L, Vyshemirskii O, Seregin S, Petrova I, Samokhvalov E, Lvov D, Gutorov V, Kuzina I, Tyunnikov G, Tang YW, Netesov S, Petrov V, 2003. Genetic analysis of Crimean-Congo hemorrhagic fever virus in Russia. *J Clin Microbiol 41:* 860–862.
27. Belousov YS, Welch RA, Sanders S, Mills A, Kulchenko A, Dempcy R, Afonina IA, Walburger DK, Glaser CL, Yadavalli S, Vermeulen NM, Mahoney W, 2004. Single nucleotide polymorphism genotyping by two colour melting curve analysis using the MGB Eclipse Probe System in challenging sequence environment. *Hum Genomics 1:* 209–217.